Functional analysis of the autophagy proteins in *Caenorhabditis elegans*

PhD Thesis

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Introduction and aims

Macroautophagy is a highly regulated self-degradative process of eukaryotic cells. During this process, parts of the cytoplasm are sequestered by a double-membrane structure, and the resulted structure is then fused with lysosomes where the delivered contents become degraded by acidic hydrolyses. The products of degradation can be reused for synthetic processes. Macroautophagy plays a role in macromolecule turnover, in particular in those of long-lived proteins, and in the removal of damaged macromolecules and organelles. Autophagy is part of the environmental and endogen stress-response pathways. Normal levels of autophagy are required for differentiation, cell growth, proliferation, ageing and response to microbial infections. Under normal conditions autophagy occurs at basal levels. The activation of this process is triggered by nutrient depletion. The TOR (target of rapamycin) kinase plays a central role in mediating starvation signals. Recently, it was found that pathways involved in tumorigenesis, such as insulin/IGF-1(insulin-like growth factor receptor-1) MAPK (Mitogen-activated protein kinase) and Ras/Raf/MAPK signaling, as well the tumor suppressor protein p53 regulate autophagy.

Genetic analysis of autophagy in multicellular organism has become possible only after the discovery of yeast autophagy genes. Since 2003, a large number of scientific articles were published on the characterization of metazoan orthologs of yeast autophagy genes. For example, in the nematode *Caenorhabditis elegans* the detection of autophagy has nearly exclusively based on the expression analysis of the reporter gene *lgg-1*/*ATG8*.

Autophagy has an essential role in macromolecular and organellar turnover. Therefore I studied the role of autophagy genes in the ageing and cell growth processes, witch are linked to protein degradation. Ageing results from the catabolic malfunction of the cells: the ageing process is caused by the intercellular accumulation of damaged macromolecules or organelles. The rate at which the tissues and cells age is regulated by evolutionary conserved pathways and various environmental factors. The proof for the genetically determination of ageing is that single gene mutations can extend lifespan dramatically. For example, in *C. elegans*, Drosophila and female mice the reduced activity of the insulin/IGF-1 receptor doubles lifespan. Autophagy is a mechanism that ensures the elimination of damaged macromolecules or organelles. Aged mitochondria or high levels of reactive oxygen species (ROS) also directly regulate the autophagic machinery through modulating Atg4 activity. Based on these results it was logic to assume that autophagy plays a central role in the regulation of ageing.

Cell growth and proliferation are closely interlinked processes: cells can divine only after they reach a critical size. The cell size is determined by homeostatic balance of the anabolic and degradative processes. Cell growth is regulated by nutrient availability and endocrine factors, including ligands for the TGF-β (transforming growth factor beta) and insulin/IGF-1 pathways. Autophagy is important for molecule turnover, and regulated by both insulin/IGF-1 and TGF-β signaling. That's way we assumed that autophagy mediates the effects of these pathways on cell growth..

1, Characterization of autophagy in *C. elegans*

For the characterization of autophagy in *C. elegans*, first I determinate the orthologs of the yeast autophagy genes by bioinformatical methods. Then I explore the autophagic process during developmental stages and in adults under normal and starvation-induced stress conditions by electromicroscopy. I also determine the localization of autophagosomes by light microscopy, using a *gfp::lgg-1* transgene and LysoTracker Red staining.

2, Assaying starvation-induced stress response in autophagy mutant nematodes

To answer the question whether autophagy genes play a role in starvation response in *C. elegans*, I performe a starvation assay on L1 larvae of autophagy mutant animals. I compare the starvation response of autophagy mutants with those of the insulin receptor mutant *daf-2(e1370)* animals.

3, Assessing the role of autophagy in the ageing process

I plan to investigate the effect of autophagic activity on the rate of the ageing process by mutational and gene silencing-based inactivation of certain autophagy genes. I analyze how compromised autophagy affects life span extension in mutant animals with inherent caloric restriction or decreased mitochondrial activity.

4, Determinating the role of autophagy genes in cell growth

To determine the role of autophagy genes in cell growth, I measure the length and body volume of certain autophagy mutant animals. I also check the cell volume in *unc-51* loss-of-function mutant animals. Furthermore, I performed an epistasis (double-mutant) analysis to see the effect of autophagy mutations on insulin/IGF-1 and TGF-β signaling mutant animals with long body size.

Materials and Method

RNA interference (RNAi)

I used RNA interference (feeding method) for inactivating certain autophagy genes. For RNAi, I used the bacterial strain HT115 that produces dsRNA specific for the gene of interest. Bacteria were previously transformed the vector pPD129.36. 3-5 larvae were transferred on induced RNAi plates at 25C, and the F1 and F2 generation were tested for phenocopy.

Electron microscopy

For fixation and embedding of transmission electron microscopic samples, the nematodes were treated individually. They were cut open under a dissecting microscope in a drop of fixative composed of 0.2% glutaraldehyde and 3.2% formaldehyde in 0.15 M cacodylate buffer. After an overnight fixation at 4°C, the fixative was changed to washing buffer (0.1 M cacodylate buffer) and the samples were embedded in agar, postfixed with 0.5% cacodylate-buffered OsO4, stained with 2% uranyl acetate, dehydrated in ethanol and propylene oxide and embedded in TAAB 4 component resin. Thereafter the samples were cut along the longitudinal body axis with a Reichert-Jung Ultracut-E type ultramicrotome, stained with lead citrate and examined using a JEM100CX II electron microscope.

Light microscopic assay

For light microscopic assay (expression pattern analysis, co-localisation or morphology examination), I used an Olympus BX-51 upright microscope. For lysosome staining, I used LysoTracker Red DND-99 (Molecular Probes, Eugene).

Starvation assay at the L1 larval stage

Embryos were isolated by hypochloric bleaching. Larvae were starved in 5 ml physiologic M9 solution. The samples were taken in every two days, and the number of survivals was determined.

Life span analysis

Nematode life span assays were carried out at 25°C. For synchronization, 20–30 gravid well-fed adults were transferred to a new agar plate containing nematode growth medium (NGM) seeded with *E. coli* OP50 to lay eggs for 4–5 hours, and then removed. F1

young adults were transferred to NGM plates supplemented with 300 mg/ml FUDR (5-fluoro-2'-deoxyuridine) and scored. Animals were considered dead when they stopped pharyngeal pumping and responding to touching. For statistic I use SSPS 14.0 program. For *p* value analysis I compared Kaplan-Maier curves with log-rank (Mantel-Cox) analysis.

Body length, volume and cell size measurements

Animals were maintained at 20°C. They were paralyzed by 0.5 M NaN_3. Body length was determined by a light microscope equipped with a scale. Cell volume and size were determined by using an Olympus BX-51 microscope. The volume was calculated from the theoretically formula $V = \pi \cdot (D/2)^2 \cdot L = (1/4) \cdot \pi \cdot A^2/L$, where D was the diameter, L was the length and A= L·D. For "seam" cell measurement, L3-stage larvae bearing an *ajm::gfp* reporter were analyzed.

Results

1, Characterization of autophagy in *C. elegans*

- 24 yeast autophagy genes we analyzed have 17 potential *C. elegans* orthologs
- *ATG4* and *ATG8* have more then one paralogs in this animal.
- Several yeast autophagy genes (*ATG11, ATG13, ATG14* and *ATG17*) have no ortologs in the *C. elegans* genome, while members of the Atg5-Atg12 complex have highly diverged nematode orthologs
- Under normal conditions, autophagic activity can be found in the hypodermis and intestine of larvae
- Inactivation of autophagy genes causes pleiotropic phenotypes ranging from superficially wild type [e.g., *atg-18(gk378), atg-7(RNAi)* and *atg-9(RNAi)* animals] to embryonic lethality [e.g., *bec-1(ok691)* mutants].
- 10-hour-long starvation in L2 stage larvae causes elevated autophagic activity in the hypodermis and gut cells, and autophagosomes become apparent also in the body wall muscle cells.
- In adult animals, starvation induces a short period of autophagic activity in the hypodermis and gut which passes away after a few hours.
- In wild-type animals and *unc-51(e369), daf-2(e1370)* and *eat-2(ad1116)* mutants, co-localization of the expression of the autophagy marker *gfp::lgg-1* with LysoTracker

Red staining specific for lysosomes shows that *gfp::lgg-1* expression in the vulval, nerve and muscle cells marks structures that are independent of autophagy.

- The twin-spots-like structure found in the lateral seam cells at the lethargus stages is a hypertrophised Golgi structure, and is labeled by different autophagy-specific reporters (*gfp::lgg-1, bec-1::gfp* and *atg-18::gfp*) and also by LysoTracker Red stain.
- Electron microscopy and co-localization assays show that the transgenic reporter *gfp::lgg-1* markes autophagosomes only in the hypodermis, gut cells and body wall muscle cells.

2, Starvation assays of L1 larvae

- L1 stage *unc-51(e369)*, *bec-1(ok691)* and *atg-18(gk378)* mutant (autophagy defective) worms have a reduced ability to survive under starvation, as compared with the wild type
- The lowered starvation tolerance of the autophagy mutant *unc-51(e369)* animals is epistatic over the starvation response of *daf-2(e1370)* mutant animals with reduced insulin/IGF-1 signaling activity.

3, The role of autophagy genes in the ageing process

- Inactivation of *atg-7* or *atg-9* autophagy genes shortens lifespan significantly, as compared with the normal animals
- Mutations in *unc-51, bec-1* and *atg-18* autophagy genes result in shortened lifespan. The „short-lived" phenotype of autophagy mutant animals is epistatic over the „long-lived" phenotype of *eat-2(ad1116)* mutants with reduced caloric intake, and of *atp-3(RNAi)* animals with reduced mitochondrial respiration.

4, The effect of autophagy on cell size

- The body length and volume of *unc-51(e369)* and *bec-1(ok691)* autophagy mutants are smaller than those found in the wild type
- The volume of the lateral „seam" cells in *unc-51(e369)* mutants is shorter than in the wild type
- The short body size of *unc-51* and *bec-1* mutant animals is epistatic over the long body size of *daf-2(e1370)* insulin receptor mutant animals and *lon-1(e185)* and *lon-2(e1678)*. TGF-β signaling mutant animals.

Conclusions

Characterization of autophagy in *C. elegans*

During my Ph.D. work, I analyzed autophagy in *C. elegans,* using electromicroscopy, transgene expression analysis and lysosomal staining method. In contrast to results provided by these different methods, I found that an integrated GFP::LGG-1 reporter developed as an autophagic marker in our lab, is effective for detecting changes in the autophagic activity, caused by genetic alternations that were already known to influences autophagy. Under normal conditions, wild-type adult nematodes accumulate GFP::LGG-1 in intestinal cells, but not in the hypodermis and muscle cells. Changes in the levels of *lgg-1* expression (disappearance or appearance/elevation) are informative about autophagy activity observed in RNAi treated or mutant animals.

Inactivation of autophagy genes results in pleiotropic phenotypes. If the mutated gene has roles in other processes beyond authophagy it can induce lethality. So that autophagy itself is not essential for the *C.elegans* viability or development.

Autophagy is required for starvation response in *C. elegans*

Autophagy plays a role in starvation response of different organisms. *unc-51(e369), bec-1(ok691)* and *atg-18(gk378)* mutant L1 stage larvae are more sensitive for starvation as wild-type animals. This lower starvation response of *unc-51(e369)* mutants is epistatic over those detected in *daf-2(e1370)* mutants. So, autophagy functions in the starvation response of the nematodes. This response is regulated downstream by the insulin/IGF-1 pathway.

Longevity pathways converge on autophagy genes to control aging in *C. elegans*

RNAi-mediated inactivation of *atg-7* and *atg-9* autophagy genes shortens lifespan which is consistent with mutational inactivation of other autophagy genes. This indicates that autophagy regulates the ageing process. Our epistatic analysis showed that the long-lived phenotype of calorically restricted *eat-2(ad1116)* mutants and *atp-3(RNAi)* animals with reduced mitochondrial respiration are hypostatic to the reduced lifespan of autophagy mutants. In summary, genetic pathways controlling lifespan, such as the nutrient sensing, mitochondrial respiratory, insulin/IGF-1 or TOR pathways function to modulate the activity of autophagy genes. So, autophagy plays a central regulatory role in animal ageing.

Autophagy genes control cell growth

Body size of *unc-51(e369)* and *bec-1(ok691)* mutant animals is smaller than that of wild-type animals. Cell size in *unc-51(e369)* mutants is also reduced, as determined by an *ajm-1::gfp* reporter system. Thus, UNC-51 and BEC-1 proteins are required for normal cell growth and body size. I also found that the small phenotype of *unc-51(e369)* and *bec-1(ok691)* mutants is epistatic over the long body length phenotype of insulin/IGF-1 and TGF-β signaling mutants. These results show that autophagy acts as a downstream effectors of the TGF-β and insulin/IGF-1 pathway to control these cell processes. In other words, TGF-β and insulin/IGF-1 pathways converge on the autophagy cascade in cell growth control.

Publications related to this PhD thesis

Papers in referred journals:

1. **Sigmond T**, Barna J, Toth ML, Takacs-Vellai K, Kovacs AL, Vellai T. (2008). Autophagy in *Caenorhabditis elegans*. *Methods Enzymol*. **451**, 521-40. (Impact factor: 2,2)
2. **Sigmond T**, Pasti G, Palfia Z, Kovács J, Kovács AL. (2008). Qualitative and quantitative characterization of autophagy in *C. elegans* by electron microscopy. *Methods Enzymol*. **451**, 467-91. (Impact factor: 2,2)
3. Tóth ML[1], **Sigmond T**[1], Borsos E, Barna J, Erdélyi P, Takács-Vellai K, Orosz L, Kovács AL, Csikós G, Sass M, Vellai T. (2008). Longevity pathways converge on autophagy genes to regulate life span in *Caenorhabditis elegans*. *Autophagy*. **21**, 4(3). 330-338. [1]contributed equally (Impact factor: 6,7)
4. Aladzsity I, Tóth ML, **Sigmond T**, Szabó E, Bicsák B, Barna J, Regős A, Orosz L, Kovács AL, Vellai T. (2007). Autophagy genes *unc-51* and *bec-1* are required for normal cell size in *Caenorhabditis elegans*. *Genetics*. **177**,, 655-60. (Impact factor: 4,2)

Lecture:

Sigmond T, Tóth ML, Borsos E, Kovács AL, Vellai T (2007). Regulation of aging by autophagy in *Caenorhabditis elegans* VII. Magyar Genetikai Kongresszus - XIV. Sejt- és Fejlődésbiológiai Napok.

Poster:

Barna J, Tóth ML, **Sigmond T**, Szatmári Zs, Pásti G, Vellai T, Kovács AL (2007). „Az Atg8/*lgg-1* gén felhasználása a makroautofágia vizsgálatára *Caenorrhabditis elegans*ban." VII. Magyar Genetikai Kongresszus - XIV. Sejt- és Fejlődésbiológiai Napok.